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(54) Title: RICE CONFERRING RESISTANCE TO ENVIRONMENTAL STRESS BY TARGETING MnSOD TO THE CHLOROPLAST

(57) Abstract: The present invention relates to the over expression of MnSOD in the Chloroplast which is the site of production of reactive oxygen species and provides the means and wherewithal of cultivating crops in areas where it would not otherwise grow normally on account of environmental stress conditions inclusive of high and low temperature, salinity, drought and ultra violet light, is resistant to herbicides thereby resulting in an increase in yield and also improved crop quality.

Rice Conferring Resistance to Environmental Stress By Targeting MnSOD to the Chloroplast

Field of the Invention

The present invention relates to the overexpression of MnSOD in the Chloroplast which is the site of production of reactive oxygen species and provides the means and wherewithal of cultivating crops in areas where it would not otherwise grow normally on account of environmental stress conditions inclusive of high and low temperatures, drought and ultra violet light, is resistant to herbicides thereby resulting in an increase in yield and also improved crop quality.

Background

Environmental stress in a broad sense is a restriction placed on living organisms by nature. The definition of environmental stress in plant science is a set of physical and chemical factors affecting the environment consequently disturbing plant growth. This stress could occur due to variant temperatures be it high or low, insufficient water supply, ultraviolet radiation and emission of pollutant gases. The study of environmental stress in plant life is significant on account of the fact that world over agricultural productivity has been greatly restricted by it and the need to withstand this kind of environmental stress is a prerequisite when studying plant life.

Under stressful conditions, the stress factor or toxic molecules derived from the stress factor attack the more sensitive molecules i.e. the primary targets in cells to impair their functions. Cells are protected by the endogenous molecular systems which mitigate the stress. The damaged targets either by having them repaired or replaced are recovered via de novo biosynthesis. When the damage caused by stress to the primary targets is very intense, the cell cannot get over the damage and the metabolic function(s) operated by the target molecules are distorted. If the repair system in respect of the damaged molecules or the energy supply system is impaired, viz, the entire cellular metabolism disintegrates allowing for the propagation of damages, then there is a cascade of events leading to cell death.

Cells have the capability of surviving stressful conditions by sensing stress and adjusting their gene expression pattern to establish new metabolism which adapt to the stress. This adaptive response is known as acclimation and it takes place from a few hours to several days in which time cells take on the stress by making use of pre existing protection systems until the new metabolism is established. The destiny of the cell is determined by the degree of available protection and the intensity and duration of the stress. The investigation of the cellular response in the early stages of environmental stress reveal the endogenous and exogenous factors that determine the stress tolerance of the plant.

The production of reactive oxygen species in cells is an inevitable restriction on aerobic life and use is made of the oxidative atmosphere for yielding energy at a high efficiency. In so far as the metabolism under non stressful conditions is concerned, reactive oxygen species is always produced. The reactive oxygen species is produced

in the cells for biosynthesis, cell defence, intra and intercellular signalling. Hence, reactive species of oxygen is both, indispensable as well as toxic to life.

It has been observed that reactive oxygen species plays a crucial role in the impairment of cellular functions due to environmental stress viz, increase in the productive of reactive oxygen species and production of oxidised target molecules under stress, decrease in the antioxidant levels or contents under stress, increased expression of the genes for anti-oxidative functions by stress, positive co-relation between the scavenging capacity for reactive oxygen species and tolerance towards stress, cross tolerance between oxidative stress and other stress.

Oxidative damage caused by reactive oxygen species can be induced by two principal mechanisms viz, an enhanced production of reactive oxygen species or by an inhibition of the scavenging systems for them. The damage proliferates production of a highly reactive hydroxyl radical and the subsequent reactions like bleaching of pigments and accumulation of oxidised lipids are apparent, these being the final symptoms of oxidative damage observed in dying cells.

Superoxide is a commonly encountered mediate of oxygen reduction. It is extremely toxic to cells since it attacks unsaturated fatty acid components of membrane lipids thereby damaging the membrane structure. Aerobic cells detoxify super oxide by the action of super oxide dismutases, metal containing enzymes that convert the superoxide radical into hydrogen peroxide and molecular oxygen. The hydrogen peroxide later converted by catalase into water and molecular oxygen.

There are three types of super oxide dismutase (SOD), copper/zinc containing SOD(CuZnSOD), manganese containing SOD (MnSOD) and iron containing SOD (FeSOD). In prokaryotic organisms MnSOD is inducible under conditions of high oxygen concentration and by O₂.

Reactive oxygen species is produced in almost every cell compartment in instances of normal metabolism (Halliwell & Gutteridge, 1989). The chloroplast containing pigments at high concentrations and evolving O₂ under light is a major source of reactive species in plant cells under illumination (Asada & Takashahi, 1987). The chloroplast and the leaf tissue is regarded as a primary site of stress induced damage in plants under light. However there are numerous cases wherein the stimuli arise from extrachloroplastic sites, e.g., the ozone, which, penetrates into the leaf tissue and interacts with apoplastic components on account of which the apoplastic antioxidant capacity assumes significance. Biotic stress like bacterial infection and grazing arises from the periphery of the cells. In the case of water stress like drought and high saline content in the soil, stress stimuli are sensed primarily by roots as well as the leaves.

In an instance where the chlorophyll (Chl) molecule at the photochemical reaction centre in the thylakoid membranes absorbs light energy, a high potential oxidative power i.e. a positive charge and a low potential reducing power, a negative charge is generated. On the oxidative side of the photosystem II (PSII) the oxidative terminus of the photosynthetic electron transport chain, water is oxidised to O₂. On the reducing side of photosystem I (PSI) the opposite terminus, the iron-sulphur protein ferredoxin (Fd) is reduced. The reduced Fd provides electrons for CO₂ fixation and other

reactions in the chloroplast. There are two potential production sites for reactive oxygen species, the reducing side of PSI and PSII.

The redox potential of the FeS centres at the terminus of PSI, ≥ 0.4 is low enough to reduce O_2 univalently to produce superoxide radical ($O_2\cdot$). $O_2 + PSI \text{ reduced} \rightarrow O_2\cdot + PSI \text{ (oxidised)}$. The photoreduction of O_2 to $O_2\cdot$ by PSI (Asada & Kiso, 1973b) inevitably occur and uses 10-20% of the photosynthetic electron flux even under conditions where CO_2 supply saturates (Asada & Takahashi, 1987). $O_2\cdot$ is disproportionate to H_2O_2 and O_2 via catalysis by superoxide dismutase (SOD) which is contained in the stroma (Asada et al., 1973).

$2 O_2\cdot + 2 H^+ \rightarrow H_2O_2 + O_2$: These reactions account for most of the photoproduction of H_2O_2 in chloroplast (Mehler reaction; Mehler, 1951). H_2O_2 is produced via non-enzymic reduction of $O_2\cdot$ with ascorbate (AsA) or glutathione (GSH).

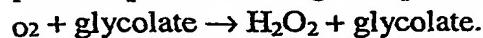
$O_2\cdot + AH \rightarrow H_2O_2 + A\cdot$, where AH and A \cdot represent either AsA or GSH and its radical, respectively. Under normal physiological conditions this mechanism is neglected since the produced $O_2\cdot$ is immediately disproportionated with SOD, which resides near the production site of $O_2\cdot$ (Ogawa et al., 1995).

O_2 photoproduced from H_2O in PSI II is finally reduced to H_2O in PSI, with catalysis by SOD and APX, to form a cycle of electron flow (water-water cycle; Asada et. Al, 1998). With regard to the produced reactive oxygen species scavenged in situ by the enzymes of the water cycle, the photoreduction of O_2 to $O_2\cdot$ is not detrimental but indispensable in preventing photoinhibition of chloroplast by acting as a safety valve that dissipates excessive excitation energy as heat (Schreiber & Neubauer, 1980, Neubauer & Yamamoto, 1992, Osmond & Grace, 1995, Laisk & Edwards, 1998). Even at 1.1 % CO_2 that saturates photoreduction of CO_2 in chloroplast, the electron flow to O_2 prevents photoinhibition despite producing $O_2\cdot$ (Park et al., 1996). This efficient scavenging of $O_2\cdot$ and H_2O_2 is ensured by high molecular activities and intraorganellar microlocalisation of the water-water cycle enzymes (Asada et al., 1998). The chloroplastic flavoenzyme monodehydroascorbate reductase has been suggested to regulate the photoproduction rate of $O_2\cdot$ at PSI (Miyake et al., 1998).

H_2O_2 is also produced outside the chloroplast not only via the disproportionation of $O_2\cdot$ but also via the divalent reduction of O_2 catalysed by various oxidases which catalyse divalent oxidation. H_2O_2 if provided with reductants and an appropriate catalyst, e.g. transition metal ions, quinones and Fd (Jacob & Heber, 1996), is reduced to form a highly toxic hydroxyl radical ($HO\cdot$) (Heber-Weiss reaction). $H_2O_2 + AH \rightarrow HO\cdot + OH^- + A\cdot$. AsA, GSH and $O_2\cdot$ can be reductants for this reaction. As catalysts the FeS centres in PSI reaction complex (Sonoike, 1996b) and in Fd (Jacob & Heber, 1996) might produce $HO\cdot$ in situ. Transition metal ions e.g. Fe, Cu and Mn, if released from metalloenzymes for some reasons are also effective catalysts. Cd from the environment also catalyses the Haber-Weiss reaction. $HO\cdot$ production is implied in the oxidative stress caused by excess Fe in tobacco (Kampfenkel et al., 1995). $HO\cdot$ can also be detected on the donor side of PS II which is impaired by UV-B (Hideg & Vass, 1996) although the source and the reaction to produce this radical is not yet known, as of now. $HO\cdot$ is highly oxidative (redox potential of $HO\cdot/H_2O$; +2.3 V) and oxidises organic molecules at the constant rate of 109 MD s $^{-1}$ (Halliwell & Gutteridge, 1989) and is toxic.

At the other end of the electron transport chain, at the time when the charges separated at the Chl dimer at the reaction centre recombine, the triplet state of Chl (3Chl) is formed and it reacts rapidly with ground state oxygen ($3O_2$) to form a singlet oxygen ($1O_2$). $3Chl + 3O_2 \rightarrow 1 Chl + 1O_2$. $1O_2$ is also produced via a similar photodynamic reaction with heme groups in proteins and with flavins through various reactions from O_2D and H_2O_2 (Halliwell & Gutteridge, 1989). In PSI II reaction centre, $1O_2$ is produced when the primary acceptor quinone QA is fully reduced (Vass & Styring, 1993). The photoproduction of $1O_2$ in PSI II has been observed *in vitro* (Macpherson et al., 1993) and *in vivo* (Hideg et al., 1998). $1O_2$ is highly reactive with organic molecules and consequently, highly toxic as well. The oxidative potential generated in the PSII reaction centre required for the oxidation of water to oxygen is potentially toxic to the PSII complex itself and damages it as a probable event (Anderson et al., 1998). The oxidant is harnessed with a charge accumulation mechanism of the Mn cluster of water oxidase (Kok et al., 1970) so as not to release the possibly generated intermediates of water oxidation, $HO\psi$, $H2O2$ and $O2D$. When water oxidase is destroyed on account of some reason or the other, such as UV-B or heat, the photogenerated oxidative power as $P680+$ or $Tyrz+$, may, oxidise the surrounding protein matrix or neighbouring molecules to inactivate PSII complex (donor-side-induced photoinhibition; Blubaugh et al., 1991, Aro et al., 1993). Further, reactive oxygen species that can be produced through photooxidation of water, may be released (Ananyev et al., 1992, Fine & Frasch, 1992, Hideg et al., 1994).

In additional chloroplastic compartments, the major production reaction for reactive oxygen species are not only the univalent reduction of O_2 to $O2D$ but the divalent reduction of O_2 to H_2O_2 . Peroxisomes contain divalent reaction oxidases and produce H_2O_2 in association with oxidative metabolisms like photorespiration and -oxidation of lipids. In C3 plants a substantial amount of H_2O_2 is produced and accompanies the photorespiration through the peroxisomal glycolate oxidase.

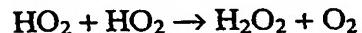
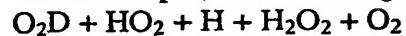


Acyl-CoA oxidase in peroxidase catalyses divalent oxidation of acyl-CoA to trans-2, 3-dehydroacyl-CoA by O_2 in the beta-oxidation of lipids, producing H_2O_2 . O_2D is produced in mitochondria. In mammalian mitochondria, O_2D production due to electron leakage from the electron transport to O_2 accounts for 1-2% of total electron flux through the chain (Chance et al., 1979) and is increased several fold by the inhibitors of electron transport, uncouplers and other agents to disrupt mitochondrial functions (Richter & Schweizer, 1997). The production of O_2D in submitochondrial particles from pea leaves has been demonstrated (Hernandez et al., 1993). Assuming that mitochondria is a major production site of O_2D in non photosynthetic cells, it has not yet been elucidated as to whether the production of O_2D in mitochondria has a physiologically positive significance as that in the chloroplast. O_2D is also produced in peroxisome and plasma membrane. In plant peroxisome, O_2D is produced via xanthine oxidase and at least three distinct NAD (P) H oxidases (del Rio, 1998). Peroxisomal O_2D production is increased during senescence and the reactive oxygen species derived from it, decompose cellular components (Brennan & Frenkel, 1977, del Rio et al., 1998). Participation in the production of O_2D of a mammalian like NADPH-oxidase on the plasma membrane in plant cells has been established upon extracellular stimuli (Auh & Murphy, 1995, Allan & Fluhr, 1997) and during lignification (Ogawa et al., 1997).

Reactive oxygen species have their respective molecular properties and reactivities with biomolecules with scavenging mechanisms for both. O₂D is generally known as a relatively stable or unreactive molecule among the reactive oxygen species. However the protonated form H₂O₂ (pK_a = 4.8) is a much higher reactive. H₂O₂ can initiate lipid peroxidation but not O₂D. Moreover H₂O₂ can pass across lipid bilayers but not O₂D. In an aqueous solution, O₂D spontaneously disproportionates to form H₂O₂ and O₂.



At a lower pH, the following reactions may occur:



The second order rate constants for these reactions are <0.35 MD1 sD1, 1.02 x 10⁷ MD1 sD1 and 8.60 x 10⁵ MD1 sD1. Since the reaction constant is the largest apparent second order rate constant for the disproportionation of O₂D, 5-105 MD1 sD1 at pH 7.0, thus decreases by 10 fold per each pH unit increase in the range over pH 5 (Bielski, 1978). O₂D is a reductant of the transition metal ions in the Haber-Weiss reaction to produce HO \cdot from H₂O₂. O₂D also propagates radical chain reaction especially in the presence of quinone. When quinones are univalently reduced to semiquinones (QH \cdot) with quinone reductases which abundantly occur in plant cells, parts of the QH \cdot reduces dioxygen to produce O₂D, which oxidises the quinols that have been produced via the disproportionation of QH \cdot to reproduce QH \cdot . This chain reaction is effectively terminated by SOD (Cadenas et al., 1992).

O₂D is highly reactive with reduced sulfur compounds like thiols and FeS cluster. O₂D oxidises thiols to the thiy radical at diffusion controlled rates (Asada & Kanematsu, 1976). The resulting thiy radicals initiate radical chain reaction. O₂D also oxidises the 4 Fe-4S cluster of aconitase in mammalian mitochondria or in bacteria at the order of 10⁶-10⁷ MD1 sD1 to the inactive (3Fe-4S) form (Radi et al., 1998). The Fe²⁺ ion released as a consequence is a potent catalyst for Haber-Weiss reaction. In plant cells the major SOD isozymes are located in chloroplasts (MnSOD). The occurrence of CuZnSOD in the apoplast and nucleus has been confirmed by immunoelectron microscopy (Ogawa et al., 1995). The occurrence of SOD implies the in situ production of O₂D. CuZnSOD and FeSOD are sensitive to H₂O₂. These SODs are the potential targets if the H₂O₂ scavenging systems do not operate properly.

H₂O₂ is a neutral, non radical molecule below pH 10 and can diffuse across biomembranes like water. The function of H₂O₂ as a stress signal (Doke, 1997) is partly based on its intra and inter cellular diffusability. H₂O₂ is a relatively weak oxidant, the oxidative potential of H₂O₂/H₂O pair is + 320 mV. However, metalloenzymes are in general sensitive targets of H₂O₂. Heme proteins can catalyse the Haber-Weiss reaction and can be degraded by the resulting HO \cdot (Puppo & Halliwell, 1998). Chloroplastic APX isozymes are inactivated by H₂O₂ in the absence of electron donors (Hossain & Asada, 1984) since compound I is irreversibly oxidised by H₂O₂ (Miyake & Asada, 1996). CuZnSOD is inactivated by H₂O₂ (Bray et al., 1974) through the reduction of Cu²⁺ ion at the reaction centre to Cu⁺ and the subsequent production of HO \cdot (Hodgson & Fridovich, 1975). CuZnSOD in isolated chloroplast of wheat leaves are inactivated by insufficient light probably due to photoproduced H₂O₂ (Casano et al., 1997). FeSOD is also inactivated by H₂O₂ (Beyer & Fridovich, 1987). The inactivation of these enzymes have been observed in vitro as

the μ M to sub-mM range of H_2O_2 , which can be reached *in vivo* as well if the H_2O_2 scavenging systems do not operate effectively. H_2O oxidises thiols to the sulfenic acids which react with thiols to form disulfides. The reaction between H_2O_2 and cysteine is slow (the apparent second order rate constant, 1MD1, sD1) but on the surface of the proteins the reaction may be largely accelerated by the presence of basic residue like Lys and Arg which could be the neighbouring thiol groups (Armstrong & Buchanan, 1978). H_2O_2 at micromolar concentrations in darkness inhibit CO_2 fixation in the chloroplast by 50% in 10 min (Kaiser, 1979) due to the oxidation of the active site thiols to the disulfide in the Calvin cycle enzymes; fructose-1; biphosphatase, NADP- glyceraldehydes-3-phosphate dehydrogenase and ribulose - 5 phosphate kinase. The activities of these inhibited enzymes are recovered by the reduction with reduced thioredoxin reversibly (Wolosiuk & Buchanan, 1977). However, if cessation of CO_2 continues under light, it will lead to excess light energy wherein the production of reactive oxygen species increases.

H_2O_2 is scavenged by two types of enzymes, catalase and peroxidase. The former scavenges H_2O_2 through the disproportionation of H_2O_2 to O_2 and H_2O corresponding to a turnover rate of about 107 minD1. (Scandalios et al., 1997). $2H_2O_2 \rightarrow O_2 + 2 H_2O$. Plants have several catalase isozymes, which are expressed in the regulated stage and tissue (Scandalios et al., 1997). Catalase is localised mainly in peroxisomes and responsible for scavenging the H_2O_2 produced in photorespiration and beta-oxidation of lipids. Catalase is a key antioxidant enzyme, a tetrameric heme containing enzyme found in nearly all the aerobic organisms which converts hydrogen peroxide into water and molecular oxygen in plants and are primarily located in peroxisomes. Plant catalases are involved in the detoxification of active oxygen species which are generated during the course of photorespiration, the beta-oxidation of fatty acids or different environmental stresses (Scandalios, 1990).

It has been shown that induction of superoxide dismutase activity in plant cells has been correlated with development of increased tolerance to a variety of chemical compounds and physical stress. Environmental stress is known to decrease crop activity according to the severity and type of stress. Enhancing tolerance of crop plants to adverse effects imposed by non optimal growing conditions for improvement of crop management. There is hence, a substantial interest in the ability to increase the concentration of super oxide dismutase in a plant cell so as to provide for a plant which has increased tolerance to environmental stress.

Summary of the invention

The present invention necessitated a comparative study of the MnSOD gene expression at mRNA level during the abiotic stress in the seedlings of contrasting *Indica* Rice varieties, IR64 and RASI, and offers a simple yet powerful tool to monitor alterations in the gene expression and further, has proved the dramatic induction of MnSOD during stress conditions. Further, the superoxide radical triggers a specific molecule in each sub cellular compartment, which is capable of acting as a signal to induce nuclear gene encoding for the particular superoxide dismutase associated with that compartment.

In the present invention, we have generated transgenic *Japonica* rice plants by co-transforming three week old scutellum callus using particle accelerator Biolistic PDS-

1000/He with the plasmid, pGV2. The plasmid pGV2 carried the MnSOD cDNA (Bowler et al, 1989), cloned downstream of CaMV promoter and the chloroplast targeting peptide followed by the NOS terminator. The pILTAB222 carried the hygromycin B phosphotransferase downstream of 35S CaMV promoter, followed by the NOS terminator.

Description of the Invention

Exposure of plants to stress conditions results in an inhibition of photosynthesis. The effects of oxygen toxicity and photoinhibition are strongly directed towards photosynthesis. Plants engineered to produce increased levels of SOD activity in response to environmental stress find use in being able to grow under conditions which inhibit growth of the parental strain, in particular conditions which increase plant super oxide to growth inhibitory levels. Examples of such conditions include salinity, drought and elevated metal concentration of the rhizosphere.

In eukaryotes, MnSOD, is a nuclear encoded protein which scavenges superoxide radicals in the mitochondrial matrix. The superoxide radicals have been ubiquitously generated in biological oxidation in all compartments of the cell. The increased production of superoxide radical is associated with a number of physiological disorders in plants. By targeting the MnSOD enzyme to the chloroplast, where the generation of superoxide is high during stress conditions, the capacity to scavenge any radical that is produced, can be increased. The chloroplast transit peptide of the small sub unit of Rubisco (ssTP) has been successfully used in both, monocotyledonous and dicotyledonous plants to target different transgenic non-plastid proteins (Cashmore et al, 1983).

The usefulness of the invention lies in the fact that it relates particularly to the use of the MnSOD gene for the protection of the plant against naturally occurring stress conditions which are not normally within the control of a farmer and as a result, the invention provides the means for growing crops in geographical areas in which they could not heretofore be grown with reasonable yields due to such naturally occurring stress conditions, including herbicide resistance, high and low temperatures, Ultra Violet light and drought.

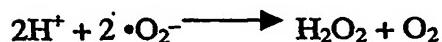
The transgenic plant is resistant or tolerant to stress conditions, particularly naturally occurring stress conditions which produce highly reactive oxygen species in one or more compartments of the plant cells, thereby increasing the potential yield and/or quality of crops produced by the plant.

After having cultured the transformants in the presence of Hygromycin, 20 different lines for molecular and biochemical analysis were selected. The presence of the gene from T₀ to T₄ generations has been proved by PCR, Southern, Northern and Western analysis. Out of the 20 lines at T₀, 17 lines were positive for Hygromycin and 11 lines were positive for MnSOD. The 11 lines that were positive for MnSOD and Hygromycin were selected for biochemical and physiological experimentation. The product of native engineered protein has been assayed and immunolocalised to the Chloroplast. Out of the 144 lines from the T₁ generation 14 lines are selected for further analysis to carry out physiological experiments for SOD and Oxidative stress.

These include herbicide resistance, high and low temperatures, Ultra Violet light and drought.

The Godawari 8 and Salween 2 rice varieties of the *Indica* rice reveal higher levels of SOD activity and SOD protein when compared to controls. Conductance measurement reflective of oxygen radical scavenging ability shows greater protection in case of transgenic Goadawari and Salween d (Fv/Fm) (change in chlorophyll fluorescence) representing chloroplast environment which in turn reveals a healthier chloroplast in the case of the transgenic Godawari 8 even under Methylviologen treatment, when compared with the control plants. The Catalase levels have been up-regulated in the transgenic line, Godawari which displayed a higher level of Godawari activity. A preliminary study of the cell viability under stress demonstrated the fact that the transgenic Godawari performed better than the controls.

To ameliorate the damage caused by hydroxyl radical formed from superoxide radical and hydrogen peroxide, organisms have evolved mechanisms to control the concentration of the two reactants. Superoxide dismutase (SOD) is a group of isozymes functioning as superoxide radical scavenger in the living organisms. Thus they protect the plant cells from superoxide radicals. The reaction of SOD is as follows:



The produced hydrogen peroxide is then detoxified by catalase or peroxidase.

In eukaryotes, the MnSOD is a nuclear encoded protein that scavenges superoxide radicals in the mitochondrial matrix. By targeting this enzyme to the chloroplast where the generation of superoxide radicals is high during stress conditions, the capacity to scavenge any radical that may be produced can be increased. In an attempt to improve stress tolerance of rice plants, an expression vector containing a *Nicotiana plumbaginifolia* MnSOD cDNA driven by a cauliflower mosaic virus 35S promoter was transferred into the TP309 *Japonica* rice callus by particle gun bombardment (using particle accelerator ballistic PDS1000/He). To target this enzyme into the chloroplast, the mature MnSOD coding sequence was fused to a chloroplast transit peptide from Pea ribulose-1-5-biophosphate carboxylase gene.

Transgenic rice *Oryza sativa* plants have been generated that overproduce the *Nicotiana plumbaginifolia L. MnSOD*. This was followed by selection and regeneration of plants. After culture in the presence of hygromycin, twenty different lines were selected for molecular and biochemical analysis. Out of twenty lines at T₀, seventeen lines were positive for hygromycin and eleven lines were positive for MnSOD. The eleven lines which were positive for MnSOD and hygromycin, were selected for biochemical and physiological experiments. At present 144 lines from the T₁ generation exist and fourteen lines are selected for further work on physiological experiments on SOD and oxidative stress. The present invention proves the presence of the gene from T₀ to T₃ generations by PCR, Southern, Northern and Western analysis.

Growth characteristics and in vitro oxidative stress tolerance of transgenic lines was evaluated how?. The transgenic lines Godawari 8 and Salween 2 showed higher levels of SOD activity and SOD protein when compared to controls (Fig 1a). Catalase levels were upregulated in the transgenic lines Godawari 8 that had higher level of SOD activity (Fig 1b). Conductance measurement reflective of oxygen radical scavenging abilities shows greater protection in case of transgenic Godawari and Salween, representing chloroplast environment shows healthier chloroplast in case of the transgenic Godawari 8 even under Methylviologen (Paraquat) treatment, when compared with control plants (Fig.1c). The presence of transgenic MnSOD activities had enhanced tolerance to Methylviologen and had increased growth rates.

Preliminary studies looking at cell viability and Root length growth assay under stress revealed that the Transgenic Godawari 8 performed better than the controls. (Figs. 2a and 2b).

The production of the native engineered protein has been assayed for expression by Northern blot analysis and is expressed in chloroplast as shown by immunolocalization studies (Figs. 3a and 3b).

The transgenic plants also exhibited considerable tolerance against oxidative damage induced by methyl viologen. The degree of abiotic stress tolerance of transgenic T(1) and T(2) plants was found to be significantly greater than that of wild-type rice plants as also measured by survival rate, chlorophyll fluorescence value, and radical elongation. The catalase activity was also highly induced in the transgenic tomato plants

. The level of H₂O₂ in the transgenic plants was lower than that in the wild-type plants under either normal or cold conditions. Results from the current study suggest that heterologous MnSOD expression in transgenic rice plants may induce several oxidative-stress responsive genes to protect from drought (abiotic) stress.

Procedure

1. Transgenic *Japonica* rice plants were generated by co-transforming 3 week old scutellum callus using particle accelerator Biolistic PDS-1000/He with plasmids, pGV2 and pILTAB222.
2. The plasmid pGV2 carried the MnSOD cDNA cloned down stream of CvMV promoter and the chloroplast targeting peptide followed by the NOS terminator.
3. The pILTAB222 carried the Hygromycin B phosphotransferase downstream of 35S CaMV promoter and followed by the NOS terminator.
4. The presence of the MnSOD gene was proved in the T₀ to T₄ generations by PCR, Southern, Northern and Western analysis

It has been found that as a result of this type of an application, there is an induction of the endogenous MnSOD expression in the chloroplast, where mostly, damaging effects of ROS occur

Dietary antioxidants are compounds in the human diet that may act to scavenge reactive oxygen species (ROS) - molecules which damage macromolecules such as DNA, carbohydrates and proteins. They play an important economic role in the processed food industry by increasing shelf life and maintaining the organo-leptic properties, vitamin content and the eye-appeal of foods.

The invention as has been stated supra, provides the means for growing crops in areas where it cannot be grown with reasonable yields on account of stress conditions including herbicide resistance, high and low temperatures, drought and ultra violet light, and, by producing a highly reactive oxygen species in one or more compartments of the plant cells, it can increase the yield and also the crop quality.

The invention, as can be gathered demonstrates the effectiveness of transformation of the plant species which provides for the expression of superoxide dismutase in a cell organelle, namely, the chloroplast. Transgenic plants are thereby generated which display increased tolerance to environmental stress.

Claims:

1. A method of enhancing the capability of a host plant to detoxify superoxide by over producing MnSOD and cultivating a whole generation of transgenic plants that confer environmental stress tolerance.
2. A method as in claim 1 wherein the superoxide dismutase is capable of transportation to a plant cell organelle.
3. A method as in claim 2 wherein the plant cell organelle is a chloroplast.
4. A method as in claims 2 and 3 wherein the MnSOD gene is targeted to the chloroplast.
5. A method as in claim 1 wherein the expression vector is pGV2 used for the transformation.
6. A method as in claim 1 wherein the promoter used is the CvMV promoter.
7. A method as in claim 1 wherein the terminator used is the NOS terminator.
8. A method as in claim 1 wherein the plasmid constructed was the pGV2.
9. A method as in claim 5 wherein the transformant was produced by transforming the vector into *Japonica* rice variety TP309 using the particle accelerator Biolistic PDS-1000/He.
10. A method whereby the regeneration of the transformed tissue comprises of the process of inducing adventitious shoots from the embryonic calli obtained from the seeds.
11. A claim as in claim 10 wherein the rooting was done from the adventitious shoots.
12. A claim as in claim 11 wherein the rooted plantlet was acclimatized to the soil.
13. A claim as in claim 1 wherein the transgenic plants could apply to all rice varieties.
14. A claim as in claim 1 wherein the transgenic plants could apply to all varieties of plants.
15. A claim as in claim 1 wherein the transgenic plant confer increased tolerance to environmental stress conditions such as drought, salinity, ultra violet radiation, heat and cold.
16. A claim as in claim 15 wherein the transgenic plants confer increased yield under environmental stress conditions.
17. A claim as in claim 1 wherein the transgenic plants will play an important role in the food industry by increasing the shelf life.
18. A claim as in claim 16 wherein the transgenic plants will maintain the organoleptic properties of foods.
19. A claim as in claims 16 and 17 wherein the transgenic plants will retain the vitamin contents in the food.

Claims:

1. A method of conferring increased tolerance to environmental stress conditions in cereal plants by overexpressing MnSOD gene.
2. A method of conferring increased tolerance to environmental stress conditions in all the rice varieties by overexpressing MnSOD gene.
3. A claim as in claims 1 & 2, wherein, the rice plant over producing MnSOD enzyme, has superior superoxide detoxification capability.
4. A method of conferring increased tolerance to environmental stress in the rice plant, by transforming cells of the said plant with DNA sequence encoding MnSOD gene.
5. A method of conferring increased tolerance to environmental stress in the rice plant, by transforming cells of the said plant with second DNA sequence encoding a transit peptide to facilitate the transportation of said MnSOD gene directed to a plant cell organelle.
6. A claim as in claims 4 & 5, wherein, the said plant cell organelle is a chloroplast.
7. A claim as in claim 5, wherein, the said transit peptide is a Pea ribulose-1,5-biphosphate carboxylase gene.
8. A claim as in claims 1 & 4, wherein, the rice plants are transformed using pGV2 as the expression vector.
9. A claim as in claims 1 & 4, wherein, the rice plants are transformed using CvMV as the promoter.
10. A claim as in claims 1 & 4, wherein, the terminator used is the NOS terminator.
11. A claim as in claims 1 & 4, wherein, the transgenic plants confer increased tolerance to environmental stress conditions such as drought, salinity, ultra violet radiation, heat and cold.
12. A claim as in claims 1 & 4, wherein, the transgenic plants confer increased yield under environmental stress conditions.
13. A claim as in claims 1 & 4, wherein, the transgenic plants confer increased tolerance to pathogen attack.
14. A claim as in claims 1 & 4, wherein, the transgenic plants will play an important role in the food industry by increasing the shelf life.
15. A claim as in claims 1 & 4, wherein the transgenic plants will maintain the organoleptic properties of foods

16. A claim as in claims 1 & 4, wherein the transgenic plants will retain the vitamin contents in the food.

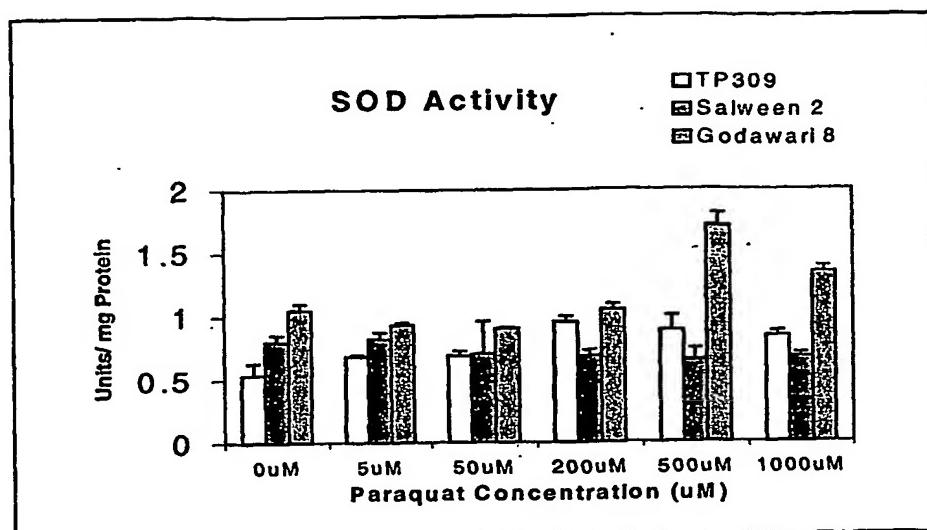
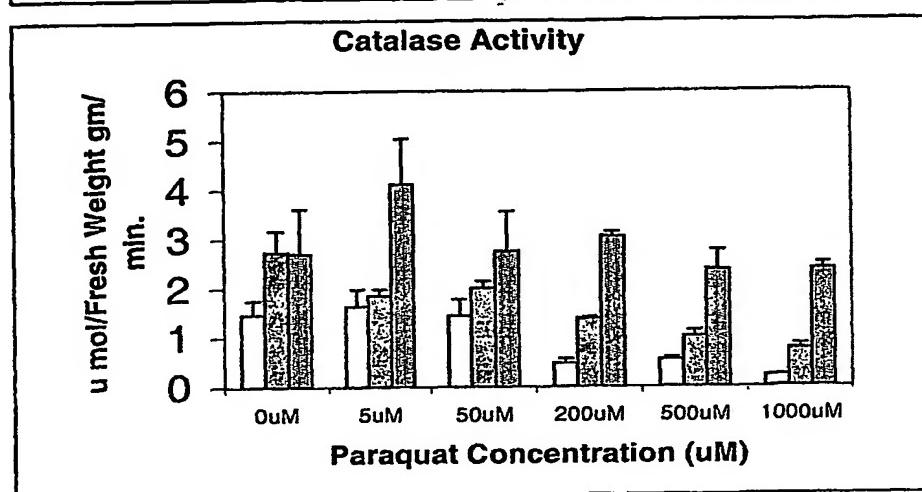
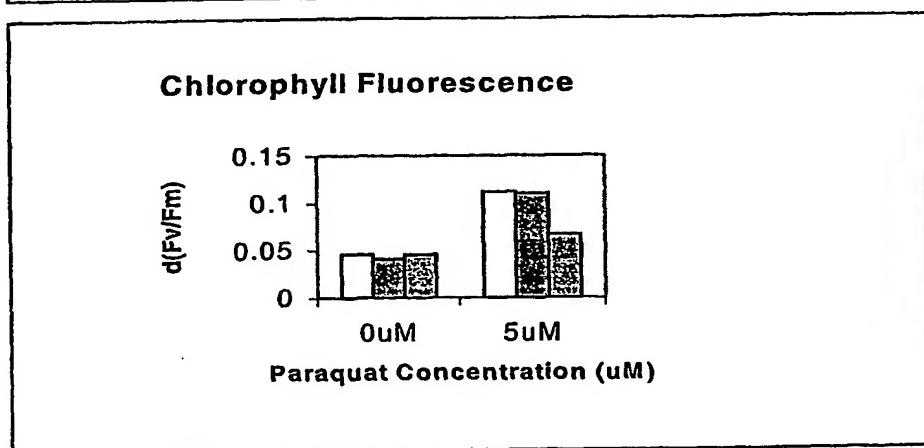
Figure 1**Figure 1a****Figure 1b****Figure 1c**

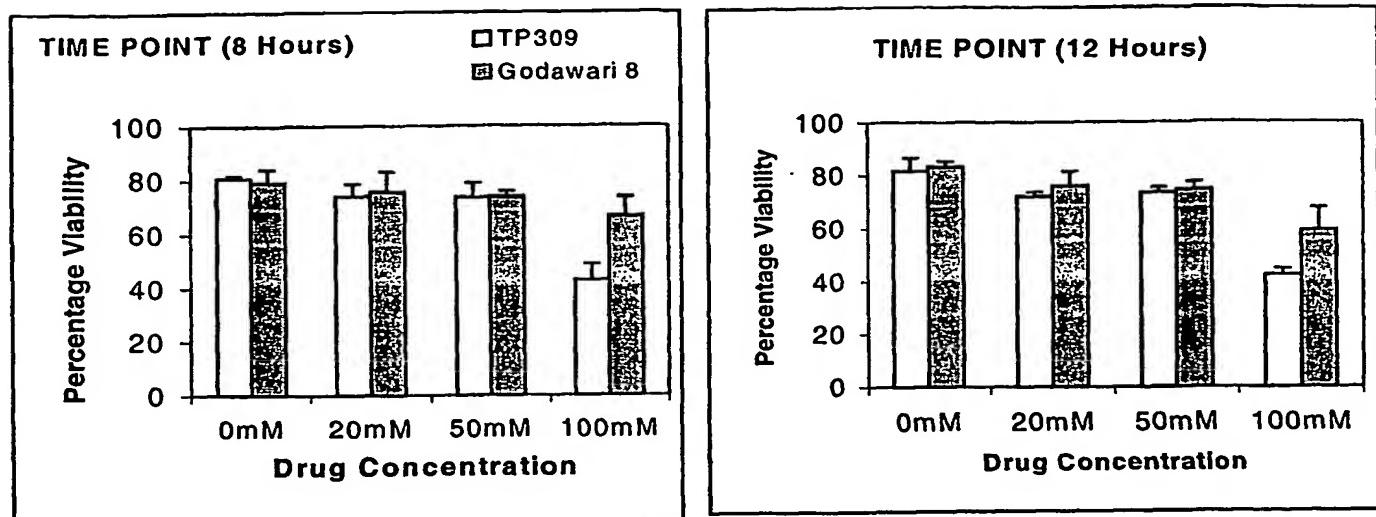
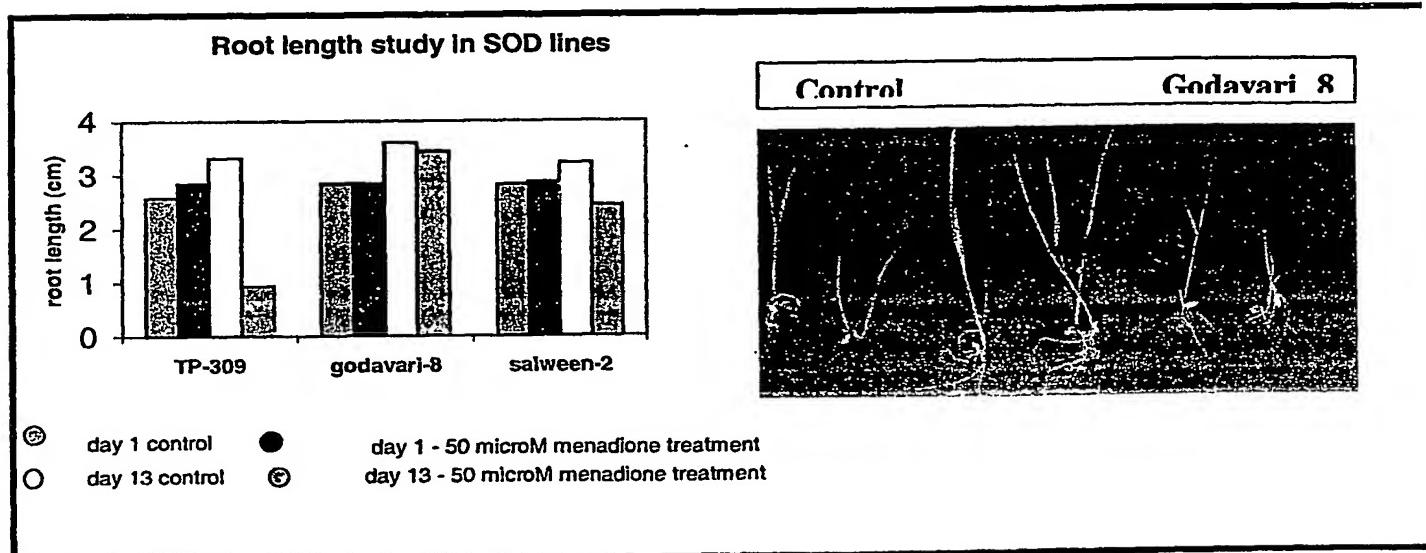
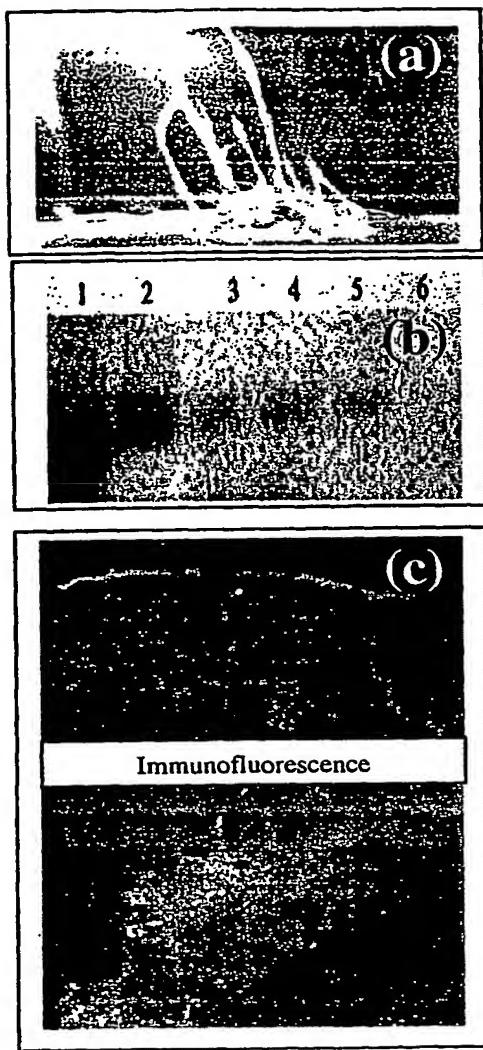
Figure 2**Figure 2a****Figure 2b**

Figure3



INTERNATIONAL SEARCH REPORT

International application No.

PCT/IB02/05253

A. CLASSIFICATION OF SUBJECT MATTER

IPC(7) : C12N 15/82, 15/90, 15/63, 15/66; A01H 5/00
 US CL : 435/ 69.1, 69.7, 468, 470; 800/278; 288, 290, 293, 295, 298, 320.2

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
 U.S. : 435/ 69.1, 69.7, 468, 470; 800/278; 288, 290, 293, 295, 298, 320.2

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
 WEST, Agricola, CAplus, Biosis

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	EP 0359617 A2 (PLANT GENETIC SYSTEMS, N.V.) 21 March 1990 (21.03.90), page 2, lines 1-35; page 4, lines 20-38 and line 63 to page 5, line 25; page 5, lines 44-59; page 6, lines 5-24 and 39-59; page 8, line 61 to page 10, line 50; page 11, lines 5-15; page 12, line 1 to page 13, line 45; page 16, line 49 to page 17, line 15.	1-4, 13-19 -----
Y		5-9 -----
X	YU et al. Waterlogging Influences Plant Growth and Activities of Superoxide Dismutases in Narrow-Leaved Lupin and Transgenic Tobacco Plants. J. Plant Physiol. 1999, Vol. 155, pages 431-438, see whole document.	1-4, 14-19 -----
Y		5-9, 13 -----
X	TANAKA et al. Salt Tolerance of Transgenic Rice Overexpressing Yeast Mitochondrial MnSOD in Chloroplasts. Plant Sci. 1999, Vol. 148, pages 131-138, see whole document.	1-4, 7, 13-19 -----
Y		5, 6, 8, 9 -----
X	VAN BREUSEGEM et al. Effects of Overproduction of Tobacco MnSOD in Maize Chloroplasts on Foliar Tolerance to Cold and Oxidative Stress. J. Exp. Bot. January 1999, Vol. 50, No. 330, pages 71-78, see whole document.	1-4, 7, 14-19 -----
Y		5, 6, 8, 9, 13 -----

Further documents are listed in the continuation of Box C.

See patent family annex.

* Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A" document defining the general state of the art which is not considered to be of particular relevance	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"E" earlier application or patent published on or after the international filing date	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"&"	document member of the same patent family
"O" document referring to an oral disclosure, use, exhibition or other means		
"P" document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search

11 July 2003 (11.07.2003)

Date of mailing of the international search report

28 JUL 2003

Name and mailing address of the ISA/US

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Authorized officer

Ashwin Mehta

Telephone No. 703-308-0196

INTERNATIONAL SEARCH REPORT

C. (Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	McKERSIE et al. Superoxide Dismutase Enhances Tolerance of Freezing Stress in Transgenic Alfalfa (<i>Medicago sativa L.</i>). <i>Plant Physiol.</i> 1993, Vol. 103, pages 1155-1163, see whole document.	1-4, 14-19
—		-----
Y		5-9, 13
X	US 5,538,878 (THOMAS et al.) 23 July 1996 (23.07.96), column 3, lines 1-50; column 4, line 28 to column 5, line 30; column 6, lines 34-52; column 7, line 18 to column 8, line 5; column 9, line 40 to column 14, line 19; column 15, line 20 to column 22, line 65.	1-4, 7, 9, 13-19
—		-----
Y		5, 6, 8
X	YU et al. Increased Tolerance to Mn Deficiency in Transgenic Tobacco Overproducing Superoxide Dismutase. <i>Ann. Bot.</i> 1999, Vol. 84, pages 543-547, see whole document.	1-4, 14-19
—		-----
Y		5-9, 13
X	VAN CAMP et al. Elevated Levels of Superoxide Dismutase Protect Transgenic Plants Against Ozone Damage. <i>Biotechnol.</i> February 1994, Vol. 12, pages 165-168, see whole document.	1-4, 14-19
—		-----
Y		5-9, 13
X	SLOOTEN et al. Factors Affecting the Enhancement of Oxidative Stress Tolerance in Transgenic Tobacco Overexpressing Manganese Superoxide Dismutase in the Chloroplasts. <i>Plant Physiol.</i> 1995, Vol. 107, pages 737-750, see whole document.	1-4, 15-19
—		-----
Y		5-9, 13, 14

INTERNATIONAL SEARCH REPORT

PCT/IB02/05253

BOX II. OBSERVATIONS WHERE UNITY OF INVENTION IS LACKING

This application contains the following inventions or groups of inventions which are not so linked as to form a single general inventive concept under PCT Rule 13.1. In order for all inventions to be examined, the appropriate additional examination fees must be paid.

Group I, claim(s) 1-9 and 13-19, drawn to a first method, of enhancing the capability of a host plant to detoxify superoxide by overproducing MnSOD.

Group II, claim(s) 10-12, drawn to a second method, of regenerating transformed tissue comprising the process of inducing adventitious shoots from embryonic calli from seeds.

The inventions listed as Groups I and II do not relate to a single general inventive concept under PCT Rule 13.1 because, under PCT Rule 13.2, they lack the same or corresponding special technical features for the following reasons: The inventions listed as Groups I-II do not relate to a single general inventive concept under PCT Rule 13.1 because, under PCT Rule 13.2, they lack the same or corresponding special technical features for the following reasons: the method of overproducing MnSOD in plants of Group I is not shared with the method of regeneration of Group II.

INTERNATIONAL SEARCH REPORT

International application No.

PCT/IB02/05253

Box I Observations where certain claims were found unsearchable (Continuation of Item 1 of first sheet)

This international report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. Claim Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. Claim Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. Claim Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of Item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:
Please See Continuation Sheet

1. As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.: 1-9, 13-19

Remark on Protest

The additional search fees were accompanied by the applicant's protest.

 No protest accompanied the payment of additional search fees.